

# VEGFR3 gene structure, regulatory region, and sequence polymorphisms

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**ABSTRACT** Vascular endothelial growth factor receptor 3 (VEGFR-3) is required for cardiovascular development during embryogenesis. In adults, this receptor is expressed in lymphatic endothelial cells, and mutant *VEGFR3* alleles have been implicated in human hereditary lymphedema. To better understand the basis of its specific endothelial lineage-restricted expression, we have characterized the *VEGFR3* gene and its regulatory 5' flanking region. The human gene contains 31 exons, of which exons 30a and 30b are alternatively spliced. The *VEGFR3* proximal promoter is TATA-less and contains stretches of sequences homologous with the mouse *Vegfr3* promoter region. In transfection experiments of cultured cells, the *Vegfr3* promoter was shown to control endothelial cell-specific transcription of downstream reporter genes. This result was further confirmed *in vivo*; in a subset of transgenic mouse embryos, a 1.6 kb *Vegfr3* promoter fragment directed weak lymphatic endothelial expression of the *LacZ* marker gene. This suggests that endothelial cell-specific elements occur in the proximal promoter, although further enhancer elements are probably located elsewhere. The sequence, organization, and variation in the *VEGFR3* gene and its regulatory region provide important tools for the molecular genetic analysis of the lymphatic system and its disorders.—Iljin, K., Karkkainen, M. J., Lawrence, E. C., Kimak, M. A., Uutela, M., Taipale, J., Pajusola, K., Alhonen, L., Halmeekytö, M., Finegold, D. N., Ferrell, R. E., Alitalo, K. *VEGFR3* gene structure, regulatory region, and sequence polymorphisms. *FASEB J.* 15, 1028–1036 (2001)

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ENDOTHELIAL CELLS (ECs) lining the blood and lymphatic vessels are dependent on receptor tyrosine kinase (RTK) -mediated signaling, which leads to growth or differentiation responses in the target cells. This signaling is required for normal development and maintenance of the vascular bed as well as for angiogenic responses in pathological conditions. Vascular

endothelial cells express nonendothelial lineage-restricted RTKs such as receptors for fibroblast growth factors and epidermal growth factors, but also endothelial cell-restricted RTKs (reviewed in refs 1, 2). Two major families consist of vascular endothelial growth factor receptors (VEGFRs) and the angiopoietin receptors, also called Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptors. VEGFRs are distinguished by an extracellular domain containing seven immunoglobulin (Ig) homology domains. The VEGFR family has three members: VEGFR-1 (also known as *flt-1*), VEGFR-2 (KDR/*flk-1*), and VEGFR-3 (FLT4) (3–7). In VEGFR-3, the fifth Ig homology domain of the extracellular part is proteolytically cleaved and the resulting polypeptides remain linked by two disulfide bonds (8). The ligands binding to VEGFRs belong to the VEGF family of growth factors, which has five cellular members: VEGF, placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D (9–13), and the recently cloned viral VEGF homologue VEGF-E (14–16). VEGFs have specific sites of expression and show distinct binding patterns to the different VEGFRs, reflecting the complex biological signaling involved in endothelial cell functions.

Targeted deletion of genes encoding VEGFRs in mice leads to impaired development of the vasculature and embryonic death (17–20). Despite the importance of the VEGFRs in the development of blood vasculature, VEGFR-3 is unique among these receptors in that it is found almost exclusively in lymphatic endothelium in adults. The expression of VEGFR-3 starts at embryonic day (E) 8.5 of mouse development in the angioblasts of the head mesenchyme, the cardinal vein, and the allantois (21). During development, however, the strongest VEGFR-3 mRNA expression becomes gradually restricted first to venous endothelia, and subsequently to the lymphatic vessels (22).

Whereas blood vessel formation has been extensively

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studied, less is known about the regulation of lymphatic vessel growth. The development of lymphatic vessels starts at E12 by centrifugal extension of vessels from venous sacs in the perimetanephric and jugular regions (20, 23). Thereafter, lymphatic vessels extend to most tissues growing around major arteries and become particularly abundant in the mesenterium and mediastinum. VEGFR-3, the first lymphatic endothelial cell-specific RTK, was cloned as an orphan receptor from human erythroleukemia cell and placental cDNA libraries (6, 7) and the *VEGFR3* gene was located to the chromosomal segment 5q33-q35 (7, 24). Subsequently, VEGF-C and VEGF-D were found to bind to and to activate VEGFR-3 (12, 13, 25). VEGF-C mRNA is expressed in close proximity to its receptor VEGFR-3 during mouse development (21). Accordingly, VEGF-C is capable of inducing hyperplasia of lymphatic vessels in transgenic mice and in chick chorioallantoic membrane (26, 27). These results suggest that VEGFR-3 plays a role in the generation of the lymphatic system.

The regulatory regions of six genes sufficient to target expression of heterologous genes specifically to the endothelial cells *in vivo* have been described. These comprise the promoters/enhancers of the *Tie1* (28–30), *Tie2* (30, 31), *Vegfr2* (32), intercellular adhesion molecule 2 (*ICAM2*) (33, 34), von Willebrand factor (*vWF*) (35, 36), and vascular endothelial cadherin (*VE-cadherin*) (37) genes. These genes are expressed in the endothelium of blood vessels, whereas the *VEGFR3* expression is relatively specific for lymphatic endothelial cells in adults except it is also expressed in some fenestrated blood endothelial cells and angiogenic blood vessels in tumors (38, 39). It is not clear which transcription factors regulate *VEGFR3* expression or what molecular mechanisms are responsible for the restriction of its expression to the lymphatic endothelium during development. Here we have cloned and analyzed the *VEGFR3* gene and characterized its structure as well as the promoter region responsible for its endothelial cell-specific expression.

## MATERIALS AND METHODS

### Genomic organization, exon–intron sequences, and polymorphisms of the human *VEGFR3*

To characterize the structure of the *VEGFR3*, a genomic cosmid clone (24) was subcloned as *EcoRI* fragments (10, 9.5, 7.0, 4.3 and 3.5 kb) into the pGEM3 vector (Promega, Madison, Wis.). The genomic clones were ordered by using 2.5, 1.2, and 0.6 kb *EcoRI* fragments of *VEGFR3* cDNA (40) as probes in Southern blotting analysis. The cosmid subclones were sequenced using primers based on the published *VEGFR3* cDNA (GenBank access. no. X68203 for VEGFR-3S and S66407 for the carboxyl-terminal tail of VEGFR-3L) (40). The sequence data from the cosmid clone was independently confirmed by using cDNA primers from the ends of exons to amplify segments of genomic DNA. We sequenced ~ 80% of the genomic DNA using PCR amplimers, cycle sequencing with dRhodamine ready reactions dye Terminator kit, and running them out on an ABI Prism Model 377. The se-

quences were analyzed further and aligned on Sequencer 3.1. (GeneCodes). Polymorphic variation in the *VEGFR3* gene was identified by resequencing of a minimum of 50 chromosomes and allele frequencies were estimated from the sequence results. Recently, a genomic region of chromosome 5 containing the *VEGFR3* gene region in unordered fragments was published (AC022095), and a direct sequence comparison confirmed our results.

### Cell culture and transfection assays

To analyze the function and activity of the *Vegfr3* promoter, luciferase reporter gene constructs were used in transfection assays of cultured cells. A 3 kb *KpnI/NodI* genomic fragment of the mouse genomic clone comprising the putative *Vegfr3* promoter was cloned into the pGL3 basic reporter vector. pGL3 basic and pGL3 control vectors contained the firefly (*Photinus pyralis*) luciferase gene without promoter and driven by the SV40 promoter/enhancer, respectively. A restriction enzyme map of the mouse 3 kb *Vegfr3* genomic fragment was made, deletions were introduced into the promoter fragment with the restriction enzymes indicated, and DNA fragments were inserted into the pGL3 basic vector. All constructs were confirmed by sequencing.

Mouse lung (LE-II) and brain endothelial cells (BEND) were grown in minimal essential medium and NIH3T3 murine fibroblasts in Dulbecco's modified minimum essential medium (DMEM) containing 10% fetal calf serum and antibiotics. Mouse keratinocytes (MK-2) were grown in low (2 mM)  $\text{CaCl}_2$  DMEM containing 10% fetal calf serum, antibiotics, and epidermal growth factor (4 ng/ml). Cells were transfected using the calcium phosphate method. The minimal amount of plasmid registering full transcriptional activity was titrated prior to the experiments. In subsequent cases, 5  $\mu\text{g}$  of the appropriate reporter construct was transfected along with 0.2  $\mu\text{g}$  of pRL-TK (Promega) to control the variability in transfection efficiency. The pRL-TK vector contains the *Herpes simplex* virus thymidine kinase promoter region upstream of the *Renilla reniformis* luciferase gene. Cell extracts were prepared 48 h after transfection in passive lysis buffer (Promega). Luciferase activity was measured using Digene DCR-1 luminometer and Promega Dual Luciferase Assay System. The ratio of firefly luciferase activity to renilla luciferase activity in each sample served as a measure of the normalized luciferase activity, which was divided by the activity of the pGL3 control vector and expressed as relative luciferase activity. Each construct was transfected at least five times and data for each construct are presented as the mean  $\pm$  SE. Relative luciferase activities among constructs were compared by a factorial analysis of variance, followed by Student's *t* test. Statistical significance was accepted at  $P < 0.05$ .

### Production and analysis of *Vegfr3* promoter-LacZ embryos

A *HindIII* fragment of mouse *Vegfr3* genomic sequence was cloned to the *HindIII* site of pSDKLacZpA. Transgenes containing either 3.6 kb *HindIII/NodI*, 1.6 kb *BsaI/NodI*, or 0.8 kb *SpdI/NodI* fragment of *Vegfr3* genomic sequence, followed by the  $\beta$ -galactosidase gene and SV40pA, were excised from the plasmid by digestion with *SalI*, *Apal*, and *BsaI/SpdI*, respectively. Transgenic DNA fragments were microinjected into fertilized oocytes of the FVB/N strain of mice and the injected zygotes were transplanted into oviducts of pseudopregnant C57BL/6xDBA/2J hybrid mice. Embryos were dissected from the decidua on day E15.5 and stained for  $\beta$ -galactosidase expression as described previously (29). The embryos were genotyped by PCR analysis of the amnion DNA

using primers 5'-TCTGTCCGATCCTT-3' and 5'-GCTGGATCGCGCGTCCGGT-3' specific for the *LacZ* gene.

## RESULTS

### Organization and sequence variation of the human *VEGFR3* gene

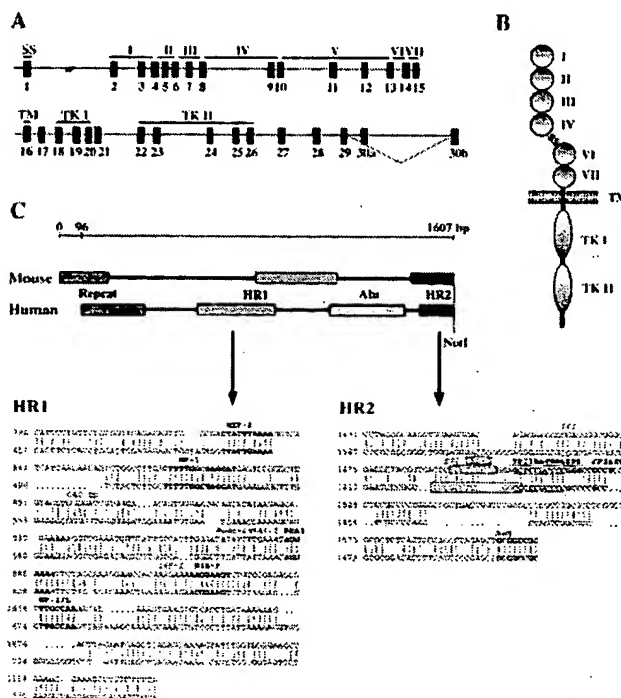
We recently found mutations in the *VEGFR3* gene in hereditary lymphedema (41, 42). Because *VEGFR3* is one of a handful of receptor tyrosine kinase genes involved in human hereditary disease, we wanted to characterize this gene and its sequence variation. A cosmid clone containing the human *VEGFR3* genomic sequences was analyzed by Southern blotting and hybridization with subcloned *VEGFR3* cDNA fragments as probes, and suitable *EcoRI* fragments were subcloned into plasmid vectors. To characterize the exon-intron boundaries, these subclones were sequenced with primers based on the published human *VEGFR3* cDNA sequence (40). The *VEGFR3* gene was found to consist of 31 exons (Fig. 1A), the largest of which (exon 13) is only 363 bp in length (Table 1). The 3' exons 30a and 30b are alternatively spliced into the mRNA, producing polypeptide isoforms differing by 65 amino acid residues (43). The nucleotide sequences of *VEGFR3* exon-intron junctions were determined in their entirety; the lengths of most of the introns were estimated by using genomic PCR and further confirmed by comparing the lengths of the PCR products to the genomic sequence covering most of the *VEGFR3* gene (Table 1). All exon-intron boundaries were found to conform to the 5'-GT-AG-3' splice site rule. The first intron of the *VEGFR3* was only partially sequenced. Its length is at least 13.5 kb whereas the rest of the coding region from the second exon to in exon 30b spans more than 29 kb of genomic DNA.

The overall genomic organization of the *VEGFR3* gene is highly similar to the previously published organization of the *Vegfr1* and *VEGFR2* genes (44, 45). The coding capacity of the different exons is indicated in Fig. 1A in relation to the domain structure of the mature VEGFR-3 receptor protein, which is presented in Fig. 1B. Resequencing of *VEGFR3* in a minimum of 25 individuals identified 22 intragenic single nucleotide polymorphisms (SNPs) and an intronic (CA)<sub>n</sub> repeat element. Six SNPs occurred in exons, two synonymous substitutions (G507T and T3198C), and four predicted to cause amino acid substitutions in the coding sequence (N149D, T494A, P641S, and R1146H). The intragenic polymorphic sites and allele frequency estimates are indicated in Table 2.

### Mouse and human *VEGFR3* promoters contain two distinct regions of homology

Sequences upstream of the mouse *Vegfr3* gene were cloned from a mouse genomic DNA library. Sequenc-

ing of clone F2N2.7, encompassing a 2.7 kb fragment upstream of the *NotI* site in the 5' leader sequence of the first coding exon, revealed that its 5'-end contains ~1.3 kb of repetitive sequences, followed by 1.4 kb of sequences not homologous to known genes (Fig. 1C). These sequences had two distinct regions of nonrepetitive sequence homology to the human promoter region that were separated by an Alu-like element in the human clone. These conserved regions, called homology regions 1 and 2 (HR1 and HR2 in Fig. 1C), showed ~70% identity between the mouse and human sequences. HR1 contained conserved putative transcription factor binding sites, including a putative site for myocyte enhancer factor 2 (MEF-2) and several possible binding motifs for transcription factors for members of the Ets family, marked PEA3 and E1A-F (Fig. 1C). HR2 was immediately upstream of the *NotI* site and contained multiple conserved binding sites for Sp1 like factors, as well as sequences that resemble the transcription start sites found in genes which do not contain a TATA box (Fig. 1C; 46).



**Figure 1.** Exon-intron structure of the human *VEGFR3* and comparison of mouse and human *VEGFR3* promoters. A) The genomic structure of human *VEGFR3* consists of 31 exons (black boxes) of which 30a and 30b are alternatively spliced. The *VEGFR3* exons encode seven Ig-homology domains (I-VII), a transmembrane region (TM), and tyrosine kinase domains (TK I and TK II), which are interrupted by the kinase insert sequence. B) Schematic structure of the VEGFR-3 receptor protein. C) Schematic structure of the upstream regions of the mouse and human *VEGFR3* genes. Repetitive sequences are marked with green boxes, homology regions 1 and 2 (HR1 and HR2) as orange and blue boxes, respectively. In the human promoter, these elements are separated by an Alu repeat. The sequences of HR1 and HR2 with conserved putative transcription factor binding sites are shown in the figure.

Table 1. Exon-intron boundaries of the *hVEGFR3*<sup>a</sup>

Exon	Bp <sup>b</sup>	Splice acceptor	5' of exon	Exon Size (CDS; <sup>c</sup> bp)	3' of exon	Splice donor	Intron size (kb)
1	20-77		ATGCAGCGG	58	TCCTGGACG	gtgagcgcggcgaacgggcccacccgccc	>13.5
2	78-174	caccacgctgacctgtctccacccacag	GCCTGGTGA	97	CTCCTGCAG	gtactgggtccctctctccactggcaggcc	0.88
3	175-419	cccacctgaccactcctgtctctgccag	GGGACAGCA	245	TCGTGAGAG	gtgagacttggaggcgggcccaggctggag	0.22
4	420-532	ttcccccaacagcttgcctcctctccatag	ACTTTGAGC	113	CTGCGCTCG	gtacggccccacccccacccagcatccg	0.12
5	533-695	gggcaatgatgggtggccttgtccctccag	CAAAGCTCG	163	ACATCACAG	gtaacagggtctgtgccccgttccagtaa	0.11
6	696-835	cgtgggagtcactgtgtgcgtgtcaacag	GCAACGAGC	140	GGGAAGCAG	gtgagggtcagcagcgtgcccaggctgtcc	0.27
7	836-1004	tgacgcggggcttccctgggtgcccggcag	GCAGAGCGG	169	TTGTGCATG	gtatggcctgggaaacagggtccttgtg	0.26
8	1005-1122	gatttaccagtgaacttctcctgtccggcag	AAAATCCCT	118	GTTCAGTG	gtaacagccctggctcctccccgaccccg	2.99
9	1123-1277	atgggcccctgacctccctccttggcag	GTACAAGCA	155	TGGTGAATG	gtagggtcagggaacgggagagcgcaggggt	0.08
10	1278-1440	cagctgaccggccatctgtgtgccacag	TGCCCCCCC	163	CGGTAGTCT	gtgagtacagctccagcctcagggtccct	1.81
11	1441-1567	cctgcccctgtccttcttgggtcctggcag	CCGGCGGCG	127	AAGAATAAG	gtacaggccagccgctgctgagtgccc	1.09
12	1568-1676	aatggatccttctgtgctgtgtctccag	ACTGTGAGC	109	ATGTACCA	gtgagtgaccagaccaggggcggggagccc	0.83
13	1677-2039	tggcacagggttcttctcctccctccacag	CCATCCCGG	363	CGGTGCAGG	gtgaggctggcccgggggaggggcggggac	0.29
14	2040-2186	cttagtccctcctgacccctgcccacag	CCCTGGAAG	147	AAAAGTCTG	gtaggagggttggccttggcgaaggaggag	0.10
15	2187-2318	gacccgcacctccacccacccctgcag	GAGTCGACT	132	CCGTGGAAG	gtctggcctcagcctgcacccctcaactc	0.16
16	2319-2425	cacacgcggggcccttcttggccacgag	GCTCCGAGG	107	ATGAGGAGG	gtgagtgtccctcctcctctgatggag	0.30
17	2426-2561	ccccactcctcctgtccctcctcccgag	CCGGCCACG	136	TGCACCTGG	gtgaggccagcaccagcctgtcccaacca	0.40
18	2562-2666	cacctgcaactggccctcctcccgacag	GGAGAGTGC	105	TGCTGAAAG	gtgtggggtcagcggggcggaggggcagc	0.31
19	2667-2780	ggccgggtgtgaggccggtgtccctccag	AGGGCGGCA	114	AGCCGCAAG	gtacggagcggcgcccgccgtgggacggc	0.14
20	2781-2869	cgcacggccgcccgcgggtcccgccgag	GCCCCCTCA	89	CCCTGCGCG	gtgagcggggcggcctgcggggcggcggg	0.10
21	2870-3020	ggcctcgagccagcttctgtgcatccgag	GAGAAGTCT	151	ACCAAGAAG	gtgagagcgtggccttctccttcttcta	1.74
22	3021-3115	tcctgagccacactggctccactgtgtag	CTGAGGACC	95	TCCCGAAAG	gtgagcttcccccgaggcccttcagacg	0.40
23	3116-3238	agcccccttgcgcctccccgcacccag	TGCATCCAG	123	AAGGGCAGT	gtgagtgcaggccattgaggagagggaac	2.66
24	3239-3350	gggggacaaggttccctctgttcccccag	GCCCGGCTG	112	TCTCTCTGG	gtgagtgcaggatgggggtgcccgtgggga	0.95
25	3351-3450	ccccaccacactcccccttctgtgtgtag	GGGCTCTCC	100	TCCCGCCAT	gtgagcctccccatggccttcaggtttt	0.40
26	3451-3556	gcagtcagcagccacgtgatactgcag	ACGCCGCAT	106	GGCCTGCAA	gtgagccccctccccacctgttctacta	1.02
27	3557-3705	ggcctggctgcttctctgttcccgccag	GAGGAAGAG	149	GGCCGCCAG	gtcagctgtcttgcagggtccagagtagg	1.30
28	3706-3826	cctcagcactctctgatttctccacag	GTATTACAA	121	GGCTCTGTG	gtacttcacatgaagggtgggggctgctgc	0.85
29	3827-3912	ccacagcctggcttgcctctccacacag	GACAACCAG	86	CGGCTTCAG	gtaagggtctctgtagcctctgcaactgc	0.68
30a	3913-	gatcatgggagcggggccttccctcttag	GTAG	4		NCS <sup>d</sup> 496 bp	4.37
30b	3913-	tactaacaccaccttccctgtcttggcag	CTGTAAAGG	199	AGCTACTAA	NCS 1923 bp	

<sup>a</sup>Intron sequences are indicated by lowercase letters and coding sequences by capital letters. <sup>b</sup>bp: basepairs of cDNA (acc. no. X68203 for VEGFR-3S and S66407 for the COOH-terminal tail of VEGFR-3L). <sup>c</sup>CDS: coding sequence. <sup>d</sup>NCS: non-coding sequence before polyA signal.

### Analysis of the mouse *Vegfr3* promoter activity in cultured cells

To analyze whether the genomic sequences 5' of the *VEGFR3* open reading frame confer endothelial cell-specific promoter activity, a 3 kb *KpnI*-*NotI* fragment upstream of the first coding exon of the mouse *Vegfr3* gene was cloned into a luciferase reporter vector and transfected into cultured endothelial and nonendothelial cells. Cell extracts were assayed for luciferase activity (Fig. 2). As the lymphatic vessels originate from the vascular endothelium and no lymphatic endothelial cell lines are currently available, blood vascular endothelial cells were used to study the *Vegfr3* promoter activity and cell type specificity *in vitro*. The *Vegfr3* expression in the endothelial cell lines used in the study was checked by Northern blot analysis. Mouse endothelial cells isolated from the lung (LE-II) did not express *Vegfr3* whereas the brain endothelial cells did (data not shown). In LE-II and BEND cells, the 3 kb *Vegfr3* promoter fragment showed 13% and 27% of the activity, respectively, of the SV40 promoter/enhancer used as a positive control. In NIH3T3 fibroblasts and MK-2 epithelial cells, the promoter showed much weaker activity (3% and 2%, respectively), indicating specificity for endothelial cells. However, the transcriptional activity of the 3 kb *Vegfr3* promoter fragment in endothelial cells was weaker than the activity of a 1.1 kb *HindIII*-*NcoI* *Tie* promoter fragment (47).

Progressive 5'-deletions were made to the *Vegfr3* promoter to characterize sequences critical for the promoter activity. Figure 2 shows schematically the deleted *Vegfr3* promoter fragments used in the transfection experiments. As only background promoter activity was detected in NIH3T3 and MK-2 cells, no conclusions about the effects of mutations to *Vegfr3* promoter activity were made based on the results obtained from these cell lines. The deletion of upstream sequences leaving only a 1.6 kb *Vegfr3* promoter fragment increased significantly (57%) the promoter activity in BEND cells, whereas in LE-II cells, the promoter activity was slightly reduced (Fig. 2). Further deletions leaving only 858 or 819 bp of the sequences upstream of the *NotI* site further increased the promoter activity in both LE-II and BEND cells. However, upon further deletion, luciferase activity driven by the 453 bp fragment was reduced by 40% in LE-II cells and 50% in BEND cells. These results suggest that the *KpnI*-*NotI* *Vegfr3* promoter fragment upstream of the HR1 contains DNA sequences inhibitory for transcription whereas the HR1 itself contains transcriptional enhancer elements.

### Activity of the *Vegfr3* regulatory sequences in transgenic mouse embryos

To analyze whether the *Vegfr3* regulatory region identified *in vitro* is functional and specific to lymphatic as

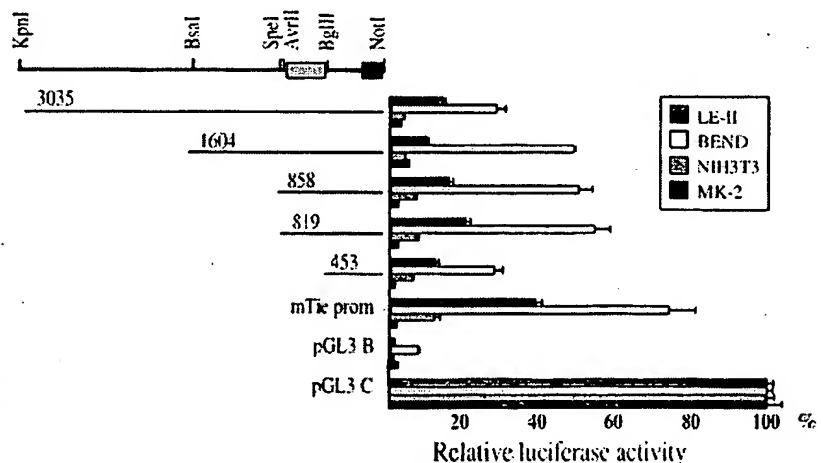
Table 2. Polymorphic variations identified in the human VEGFR3 and the conditions for genotyping these polymorphisms

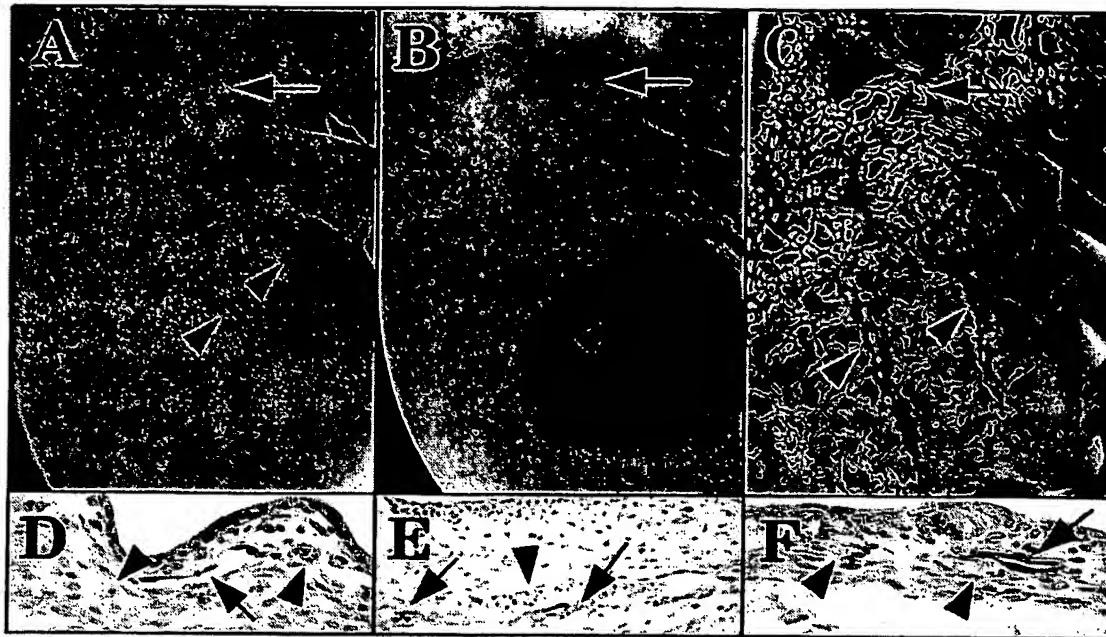
Location	Nucleotide	Detection method	Forward primer	Reverse primer	Annealing Tm (°C)	MgCl <sub>2</sub> (mM)	Allele frequencies
Int3-40 bp 3' to ex3	C to T	<i>SmaI</i>	ccagctccctacgtgttcg	ggcaacagctggatgtca	56	1.0	C = 0.75 T = 0.25
Int3-55 bp to ex3	C to T	<i>ApaI</i>	"	"	"	"	C = 0.88 T = 0.12
Int3-185 bp 3' to ex3	G to A	DNA sequencing	"	"	"	"	G = 0.97 A = 0.03
Int3-200 bp to ex3	G to C	<i>AfuI</i>	"	"	"	"	G = 0.75 C = 0.25
Ex4 (N149D)	A445G	<i>SstI/AclI</i>	"	"	"	"	A = 0.99 G = 0.01
Ex4 (silent)	G507T	<i>HhaI</i>	"	"	"	"	G = 0.63 T = 0.37
Int7-65 bp 3' to ex7	G to A	<i>SstI</i>	ctgtgagggcggtggaggt	gtcctttgagccactcga	54	1.5	G = 0.48 A = 0.52
Int7-215 bp 3' to ex7	C to T	DNA sequencing	"	"	"	"	C = 0.78 T = 0.22
Int9-23 bp 3' to ex9	C to T	<i>HhaI</i>	ccagcacaggcacctacacc	agtgcactggatgctgaga	58	1.5	C = 0.90 T = 0.10
Ex11 (T494A)	A1480G	<i>HhaI</i>	ggtagctggatatgacaag	gcctacagactgcaggaa	58	1.5	A = 0.90 G = 0.10
Ex13 (P641S)	C1921T	DNA sequencing	tcaccatcgaatccaagc	agttctgcgtgagccgag	56	1.0	C = 0.99 T = 0.01
Int15-82 bp 3' to ex15	C to G	DNA sequencing	ccttgggcaagtcgtggc	gagagagactccatcagg	54	1.5	C = 0.92 G = 0.08 (CA) <sub>9</sub> = 0.79, (CA) <sub>10</sub> = 0.04, (CA) <sub>11</sub> = 0.17
Int15-118 bp 3' to ex15	CA repeat	Polyacrylamide gel	"	"	"	"	
Int17-238 bp 3' to ex17	A to C	<i>HhaI</i>	catcaagacgggtaccc	ccgctgacccacacccct	56	1.0	A = 0.72 C = 0.28
Int17-287 bp 3' to ex17	C to T	DNA sequencing	"	"	"	"	C = 0.95 T = 0.05
Int17-333 bp 3' to ex17	C to T	<i>BspI286I</i>	"	"	"	"	C = 0.88 T = 0.12
Int22-169 bp 3' to ex22	G to T	<i>SstI</i>	gaagctgaggacctgtgg	gcggacgtagtcagggtc	56	1.0	G = 0.74 T = 0.26
Ex23 (silent)	T3198C	DNA sequencing	gagttgacctcccaaggt	tctcttgagcaggcagtc	56	1.5	T = 0.99 C = 0.01
Int24-134 bp 3' to ex24	T to C	DNA sequencing	gcagagtgcagctgtggc	gccctcatccttgtgtccg	58	1.5	T = 0.86 C = 0.14
Int24-673 bp 3' to ex24	A to G	DNA sequencing	"	"	"	"	A = 0.58 G = 0.42
Ex26 (R1146H)	G3437A	<i>SfaNI</i>	cggcacaaggatgagggc	aatgcaggctctgccttg	58	1.5	G = 0.84 A = 0.16
Int28-586 bp 3' to ex28	T to C	DNA sequencing	gctgagaccctgtggtcc	agctcaccttgaacgcgc	54	1.0	T = 0.73 C = 0.27
Int29-616 bp 3' to ex29	G to A	DNA sequencing	"	"	"	"	G = 0.65 A = 0.35

opposed to blood vascular endothelium *in vivo*, we generated transgenic mouse embryos having the 3.6 kb *HindIII-NotI* promoter fragment upstream of the *LacZ* reporter gene. The embryos were stained and analyzed for  $\beta$ -galactosidase expression at E15.5. At this developmental stage, the lymphatic vessels are developing, while the skin is still permeable to the  $\beta$ -galactosidase staining reagents. Of 11 embryos positive for the 3.6 kb *Vegfr3* promoter/*LacZ* DNA, endothelial-specific trans-

gene expression was observed in only one (Fig. 3A). Three other embryos with staining in a subset of endothelial cells also showed some ectopic expression. Two DNA-positive embryos showed only ectopic staining and five did not stain at all. As the 3.6 kb *Vegfr3* promoter fragment could occasionally target expression of the *LacZ* reporter gene to the endothelium of mouse embryos, we tested whether the 1.6 kb *BsaI-NotI* promoter fragment could drive the reporter gene ex-

**Figure 2.** Activities of mouse *Vegfr3* promoter fragments used in transfected endothelial and nonendothelial cells. Restriction enzyme cleavage sites used in making the deleted constructs are shown along the *KpnI-NotI* promoter fragment. HR1 and HR2 are marked as orange and blue boxes, respectively. The lengths of the promoter fragments are indicated as base pairs above each construct. The promoter activities were analyzed in LE-II and BEND endothelial cell lines. NIH3T3 fibroblasts and MK-2 epithelial cells were used as a nonendothelial cell controls. Promoter activity is presented as percentage of the pGL3 control, and the Tie-1 promoter activity is indicated for comparison.





**Figure 3.** Expression of the mouse *Vegfr3* promoter constructs in 15.5 day p.c. mouse embryos. Comparison of the staining patterns obtained with the 3.6 kb (A) and 1.6 kb *Vegfr3* promoter/*LacZ* (B) transgenes with that of the *Vegfr3*/*LacZ* knock-in embryos (C). Note that the staining pattern is the same in all panels, although weaker in the case of the transgenes. The staining of a branching network is particularly evident in the neck region (arrows) and in the lateral regions of abdomen (arrowheads). Analysis of the skin sections from the corresponding embryos (D–F) indicates that the  $\beta$ -galactosidase expression is restricted to the lymphatic vessels (arrows). No  $\beta$ -galactosidase activity was found in the adjacent blood vessels (arrowheads).

pression specifically to the lymphatic endothelium. Based on whole mount analysis, of the 22 embryos positive for the 1.6 kb *Vegfr3* promoter/*LacZ* transgene DNA endothelial-specific transgene expression was observed in two, while seven embryos had transgene expression also in nonendothelial cells. One of the two embryos having endothelial cell-specific expression is shown in Fig. 3B. As can be seen from the Fig. 3A, B, the  $\beta$ -galactosidase marker was expressed in a loose branching network pattern in the skin in both cases. This pattern was particularly evident in the dorsal parts of the developing forelimbs and in the neck region. The morphology of these structures suggested that they represent developing lymphatic vessels that form in the skin at this stage of mouse development by an (lymph)angiogenic mechanism. A similar kind of staining pattern, although much stronger and more extensive, was seen in the *Vegfr3* *LacZ* knock-in embryos (Fig. 3C). No blue staining could be found in similarly treated transgene-negative embryos.

The vascular specificity of the staining patterns was confirmed by analyzing tissue sections. Skin sections of the 3.6 kb *Vegfr3* promoter/*LacZ* transgenic embryo revealed that although the  $\beta$ -galactosidase staining was very faint, it was clearly restricted to the developing lymphatic endothelium (Fig. 3D). Analysis of the sections from the 1.6 kb *Vegfr3* promoter/*LacZ* transgenic embryos confirmed that this fragment was sufficient to drive  $\beta$ -galactosidase expression largely to the lymphatic vessels (Fig. 3E), although some veins were also weakly positive. However, the other transgenic embryo for the 1.6 kb *Vegfr3*/*LacZ* construct, which showed

staining only in the lymphatics, had some weak staining in the chondrocytes. Analysis of tissue sections of the *Vegfr3*/*LacZ* knock-in embryo confirmed the endogenous pattern of expression and weaker activity of the promoter fragments (Fig. 3F). Staining of blood capillaries or arteries was not observed in either case.

As the 1.6 kb promoter fragment was able to drive the gene expression to the lymphatic endothelium in some of the embryos, we continued to study the reporter gene expression targeted by the 0.8 kb *Sp1-NotI* *Vegfr3* promoter fragment, which showed stronger promoter activity in cultured cells. In the transgenic analysis of the 0.8 kb *Vegfr3* promoter/*LacZ* construct, 20 embryos screened were transgene positive; of these, four embryos had staining in endothelial cells. Three also had ectopic staining in other cell types. However, even in the embryos showing endothelial cell-specific expression of the transgene, the staining pattern was not restricted to the developing lymphatic endothelium. Strong promoter activity was found also in the developing capillary-sized vessels, e.g., in the head (data not shown). Table 3 summarizes the results from the *Vegfr3* promoter/*LacZ* transgenic studies.

## DISCUSSION

In this study, we have characterized the structure of the human *VEGFR3* gene and both mouse and human *VEGFR3* promoters. The coding sequence of the *VEGFR3* gene is organized into 31 exons that closely



Table 3. Summary of the *in vivo* activity of *Vegfr3* promoter fragments in transgenic mouse embryos at E15.5

Promoter fragment	TG <sup>a</sup>	ES <sup>b</sup>	ES/ET <sup>c</sup>	ET <sup>d</sup>	NO <sup>e</sup>
3.6 kb <i>HindIII/NotI</i>	11	1	3	2	5
1.6 kb <i>Bsal/NotI</i>	22	2 <sup>f</sup>	7	0	13
0.8 kb <i>SpeI/NotI</i>	20	1	3	1	15

<sup>a</sup>TG: number of transgenic embryos. <sup>b</sup>ES: embryos showing LacZ staining only in endothelial cells. <sup>c</sup>ES/ET: number of embryos showing ectopic and endothelial staining. <sup>d</sup>ET: number of embryos showing only ectopic staining. <sup>e</sup>NO: number of embryos with no staining. <sup>f</sup>Weak staining was observed in a subset in chondrocytes in one of the embryos.

correspond to the genomic organization of the mouse *Vegfr1* and human *VEGFR2* genes (44, 45). In our analyses, we also identified many polymorphisms of the *VEGFR3* sequence. The data concerning the exon-intron boundaries and polymorphisms is important and useful from the clinical point of view as we have recently demonstrated that *VEGFR3* missense mutations are a cause of familial early onset lymphedema (41, 42). Due to the complexity and heterogeneity of this disease, it is likely that several other *VEGFR3* mutations may also be found in different lymphedema families. Using the information about the *VEGFR3* genomic structure, intragenic polymorphisms and linkage studies in families with lymphedema or other inherited abnormalities of the lymphatic system should allow rapid screening for mutations in *VEGFR3*-linked families. Since the endothelial cell-associated genes are candidate loci for vascular dysmorphogenesis syndromes and hereditary angiogenic disorders, analysis of *VEGFR3* mutations from patients suffering from lymphatic disorders might reveal the molecular basis of such phenotypes.

We have also isolated and partially characterized the genomic regions upstream of the mouse and human *VEGFR3* genes. The upstream sequences showed ~70% identity in two distinct regions, which represented the putative 5' enhancer/promoter and 3' minimal promoter elements, respectively. No classical TATA or CAAT boxes were found in *VEGFR3*. The 3 kb fragment of the 5'-flanking sequence of the mouse *Vegfr3* demonstrated activity in cultured endothelial cells but only minimal activity in keratinocytes and fibroblasts. Deletion studies of the *Vegfr3* promoter indicated that both homology elements were needed for activity. The 5' homology element had conserved putative binding sites for transcription factors conforming to the MEF-2, NF-1, AP-2, GATA, and Ets families. Two Ets family transcription factor binding sites, PEA3 and E1A-F, were conserved in the mouse and human *VEGFR3* promoters. Sites for Ets-related transcription factors are also found in all other known promoters showing endothelial specificity *in vivo*, such as in the promoters of genes encoding Tie-1, Tie-2, VE-cadherin, ICAM-2, and vWF (29–31, 33, 35–37). The 3' homology region contains multiple conserved binding sites for Sp1 like factors as well as sequences that resemble the transcrip-

tion start sites found in genes that do not contain a TATA box. The transcription factors controlling the *VEGFR3* gene expression remain unknown and further studies are needed to determine which consensus binding sites are critical for the *VEGFR3* promoter activity.

We also studied the ability of three *Vegfr3* promoter fragments to target gene expression in the developing lymphatic endothelium *in vivo*. Our results from transgenic embryos at E15.5 indicate that the 3.6 kb and 1.6 kb *Vegfr3* promoter fragments contained specific elements sufficient to direct gene expression to the lymphatic endothelium. However, as only few of the transgenics showed a similar staining pattern to that of the *Vegfr3/LacZ* knock-in embryos, the gene expression driven by these promoter fragments is apparently very sensitive to interference by the transgene integration site and further enhancer elements needed for reproducible endothelium-specific reporter gene expression are likely to be missing from these promoter fragments. Many tissue-specific gene regulatory elements are located within the first two introns, although such elements occasionally can be found at a great distance, even in the 3' parts of genes. *Vegfr2* and *Tie2* are examples of endothelial cell-specific genes whose activity is partly regulated by enhancers located in their first introns (31, 32). Therefore, we are currently analyzing the large first introns of the mouse and human *Vegfr3* genes to locate putative enhancer elements. We have sequenced the first intron of the mouse *Vegfr3* consisting of 15 kb of genomic DNA. When compared with the human *VEGFR3* first intron, which has recently become available in the GenBank, the sequences were found to be ~70% homologous. Several short regions showing high homology were found, but so far no additional enhancers could be identified in transfection studies using various DNA fragments from the mouse intron (unpublished data of the authors).

Lymphatic vessels are quite different from arteriae, veins, and capillaries in structure. The lymphatics are characterized by an extremely permeable, thin endothelial lining devoid of a basal lamina. In addition, the small lymphatic vessels typically lack supporting cells, such as pericytes or smooth muscle cells (21, 48). These differences indicate that the lymphatic endothelial cells represent a differentiated form of endothelial cells. Differentiation of endothelia to arterial, venous, and lymphatic lineages has not been understood at a molecular level. Recent evidence suggests that arteries and veins are genetically determined at the earliest stages of vasculogenesis (49, 50). It is unclear at precisely what stage lymphatic vessels are determined. One possibility is that VEGF-C and VEGF-D expressed by smooth muscle cells of arteries and veins would be involved in directing the formation of lymphatic vessels around them (38). The isolation of the *VEGFR3* promoter now makes it possible to identify the transcription factors that contribute to lymphatic development using techniques of mouse molecular genetics. The relevant transcription factor binding sites in addition to the

coding sequence may also be targets of mutations in human lymphedema.

Previous studies have aimed to identify promoter elements necessary for uniform vascular endothelial cell-specific gene expression. However, a major obstacle in gene therapy directed to endothelial cells is the fact that leakage of catheters and blood flow rapidly washes away delivery vehicles, resulting in undesired gene expression in distant organs, particularly in the liver (51). Therefore, promoters that restrict expression of transgenes to particular subsets of endothelial cells may be more desirable in such settings. In hereditary lymphedema with reduced *VEGFR3* signaling in heterozygous affected individuals (41, 42), genes that would induce VEGFR-3 signaling specifically in the lymphatic endothelium might improve the growth and function of lymphatic vessels without side effects in other tissues. Another potential use for the *Vegfr3* promoter would be in 'partial' rescue experiments, as null mutations in several endothelial-specific genes of mice are known to lead to embryonic lethality at an early stage. Although the transcriptional activity of the *Vegfr3* promoter is relatively weak, identification of additional endogenous enhancers or the use of enhancers increasing promoter activity without losing its cellular specificity might increase the efficacy of *Vegfr3* promoter in the potential applications described above (52).

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